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ORIGINAL ARTICLE

Molecular Mapping of Four Blast Resistance Genes using Recombinant Inbred Lines of 93-11 and Nipponbare

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Abstract Molecular mapping of new blast resistance genes is important for developing resistant rice cultivars using marker-assisted selection. In this study, 259 recombinant inbred lines (RILs) were developed from a cross between Nipponbare and 93-11, and were used to construct a 1165.8-cM linkage map with 131 polymorphic simple sequence repeat (SSR) markers. Four major quantitative trait loci (QTLs) for resistance to six isolates of *Magnaporthe oryzae* were identified: *qPi93-1*, *qPi93-2*, *qPi93-3*, and *qPiN-1*. For the three genes identified in 93-11, *qPi93-1* is linked with SSR marker RM116 on the short arm of chromosome 11 and explains 33% of the phenotypic variation in resistance to isolate CHE86. *qPi93-2* is linked with SSR marker RM224 on the long arm of chromosome 11 and accounts for 31% and 25% of the phenotypic variation in resistance to isolates 162-8B and ARB50, respectively. *qPi93-3* is linked with SSR marker RM7102 on chromosome 12 and explains 16%, 53%, and 28% of the phenotypic variation in resistance to isolates CHE86, ARB52, and ARB94, respectively. QTL *qPiN-1* from Nipponbare is associated with SSR marker RM302 on chromosome 1 and accounts for 34% of the phenotypic variation in resistance to isolate PO6-6. These new genes can be used to develop new varieties with blast resistance via marker-aided selection and to explore the molecular mechanism of rice blast resistance.

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Introduction

Blast is one of the most destructive diseases of rice worldwide because of the high variability of the causal agent, the fungus *Magnaporthe oryzae*. The use of host resistance is a cost-effective and environmentally responsible approach for the control of the disease. Over the last three decades, rice breeders have extensively used resistance (*R*) genes, which confer resistance to *M. oryzae* isolates containing the corresponding avirulent genes, in rice breeding programs to develop cultivars with broad-spectrum resistance. Major *R* genes, however, are often rapidly overcome by new, virulent isolates.

Genetic studies of resistance to rice blast began in the early 1960s (Ou 1985). To date, at least 85 major *R* genes have been identified and molecularly mapped on the rice linkage map (Ballini, 2008; Liu, 2010). The identified blast *R* genes are located on all 12 chromosomes except chromosome 3, and among of them cluster in particular regions on chromosomes 6, 11 and 12. Among the mapped *R* genes, 19 have been cloned (Tabien et al. 2004; Talukder et al. 2004; Chen et al. 2005; Ballini et al. 2008; Liu et al. 2010). Except *Pi-d2*, all *R* genes encode nucleotide-binding site and leucine-rich repeat (NBS-LRR) proteins, suggesting that a conserved defense mechanism may exist in rice to defend against pathogens.

The two major subspecies of cultivated rice (*Oryza sativa* L.), *indica* and *japonica*, differ in their resistance to rice blast (Shang et al. 2009). The genomes of the *japonica* cultivar Nipponbare and *indica* cultivar 93-11 were sequenced over 10 years ago (Goff et al. 2002, Yu et al. 2002; International Rice Genome Sequencing Project 2005). The availability of these genome sequences has facilitated the molecular mapping and positional cloning of useful genes in rice (Sakaki et al. 2005; Xu et al. 2005). Nipponbare harbors the resistance gene *Pia* on chromosome 11 (Yaegashi et al. 1983) and *Pish* on chromosome 1 (Imbe and Matsumoto 1985) and is resistant or moderately resistant to race PO6-6 of the blast fungus (Takehisa et al. 2009). The popular hybrid rice restorer line 93-11 confers resistance to many Chinese isolates of *M. oryzae* (Yang et al. 2009), and one blast *R* gene, *Pi41,* has been identified in the line (Yang et al. 2009).

In the present study, a recombinant inbred line (RIL) population derived from a cross between Nipponbare and 93-11 was developed for the mapping of *R* genes from both parents. A genetic map consisting of 1165.8 centimorgans (cM) was constructed with 131 polymorphic markers. Four major *R* genes were identified that confer resistance to six *M. oryzae* isolates.

Results

Characterization of Resistance of the Parental Cultivars and RILs to *M. oryzae* Isolates

Inoculation with *M. oryzae* showed that both 93-11 and Nipponbare were resistant to isolate 0-249 but susceptible to isolates (race) 82C7, RB22, and RB18. Because the two parents responded similarly to these isolates, these isolates were not used for the inoculation of the RILs. 93-11 was resistant but Nipponbare was susceptible to isolates CHE86, 162-8B, R01-1, R01-3, KJ201, 97-104-2, ARB52 (IA-1), ARB94 (IB-1), and ARB50 (IC-1). In contrast, Nipponbare was resistant but 93-11 was susceptible to isolate PO6-6 (Table 1, Fig. 1). For inoculation of the RILs, we selected the following six isolates that elicited clearly different disease

Table 1. Reactions of the parental rice cultivars to 11 isolates of *M. oryzae*

Isolate	Origin	9311	Nipponbare
R01-1	Korea	R[a]	S
162-8B	China	R	S
PO6-6	Philippines	S	R
0-249	China	R	PR
R01-3	Korea	PR	PS
KJ201	Korea	PR	S
CHE86	China	R	S
82C7	China	S	S
97-104-2	China	PR	S
RB22	China	S	S
RB18	China	S	S
ARB52	US	R	S
ARB94	US	R	S
ARB50	US	R	S

[a]R=Resistant, S=Susceptible, PR=Partial resistant, and PS=Partial susceptible

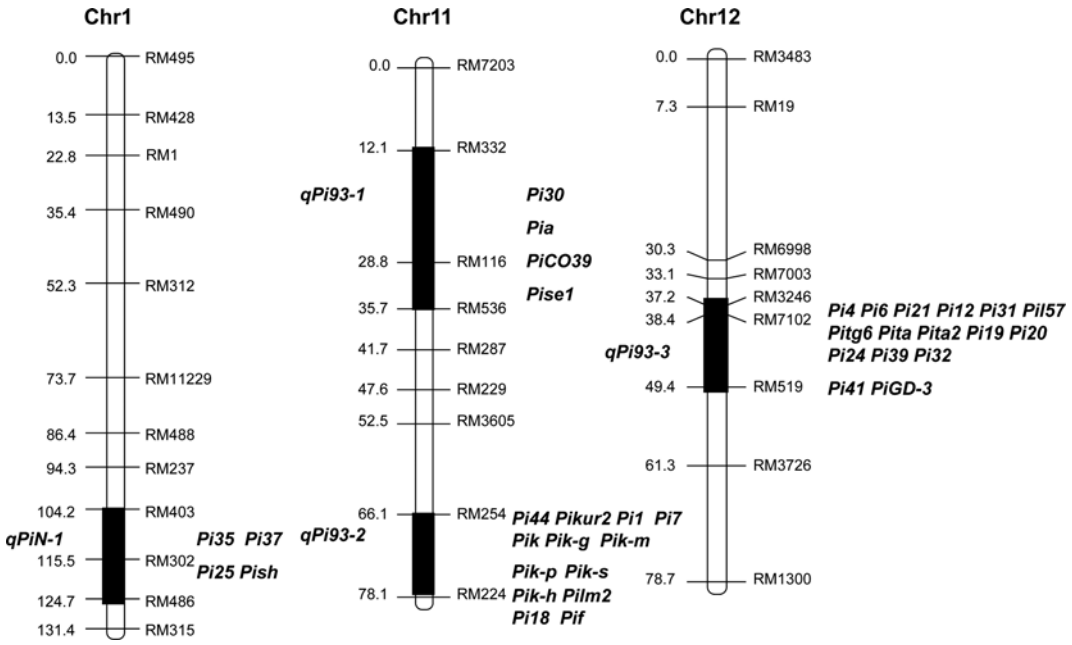

Fig. 1. Chromosome locations of the four newly-identified genes conferring resistance to *Magnaporthe oryzae* in rice. Known resistance genes located near the newly identified genes are also indicated.

Table 2. Segregation ratio of resistant and susceptible lines in the RILs inoculated with *M. oryzae*

	No. of RILs		Ratio of resistant to susceptible lines		
Isolate	Resistant	Susceptible	Observed	Expected	X^2 for expected ratio[a]
PO6-6	80	89	0.9:1	1:1	0.38[ns]
CHE86	160	31	5.2:1	7:1	2.10[ns]
162-8B	52	49	1.1:1	1:1	0.04[ns]
ARB52	137	115	1.2:1	1:1	1.75[ns]
ARB94	115	133	0.9:1	1:1	1.17[ns]
ARB50	149	101	1.5:1	1:1	8.84**

[a]– [ns]Not significant at 0.05; **significant at 0.01

reactions from the two parents: CHE86, 162-8B, ARB52, ARB94, ARB50, and PO6-6.

A total of 210, 124, 252, 248, 250, and 195 RILs were inoculated with isolate CHE86, 162-8B, ARB52, ARB94, ARB50, and PO6-6, respectively. When plants were inoculated with CHE86, the segregation ratio of resistant to susceptible plants was 7:1 (χ^2 =2.1< $\chi^2_{0.05}$ =3.84) (Table 2). This ratio indicated that three *R* genes control the resistance of 93-11 to CHE86. For PO6-6, the ratio of resistant to susceptible plants was 1:1 (χ^2 =0.38), indicating that one *R* gene confers resistance to PO6-6 in Nipponbare. For 162-8B, ARB52, and ARB94, the segregation ratio of resistant to susceptible plants was 1:1 (χ^2=0.04, 1.75, 1.17), indicating that one *R* gene confers resistance to these isolates in 93-11. For ARB50, the segregation ratio of resistant to susceptible plants was 1.5:1, which did not fit the 1:1 ratio of one dominant R gene segregation.

Construction of a Linkage Map

The genotype data of the 131 SSR markers in the 259 RILs were used to construct a linkage map (Fig. 2). With an average of 8.9 cM between each SSR marker, the mapped markers were evenly distributed on the 12 rice chromosomes with a total map length of 1165.8 cM. Among the SSR markers, 38.9% (51 of 131 loci) showed significant distortions

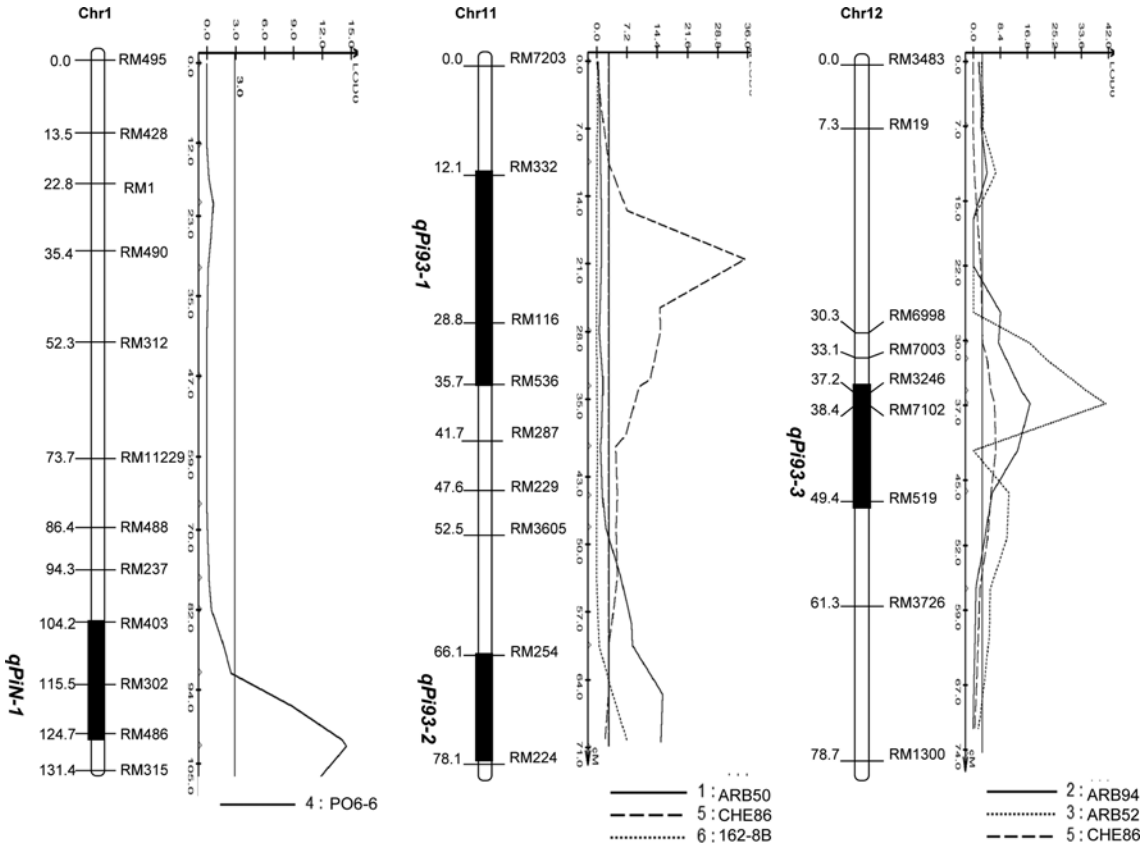

Fig. 2. QTL mapping results and linked markers of the four blast resistance genes in rice.

Table 3. Locations of the four newly identified genes that confer resistance to *M. oryzae* in the Nipponbare/9311 RILs

Resistant-					Phenotypic			
Isolate	Parent	R gene	Chr.	Marker interval	Nearest marker	LOD Value	variation explained (%)	Additive effect
PO6-6	Nipponbare	*qPiN-1*	1	RM403-RM486	RM302	15.89	34	0.36
CHE86	9311	*qPi93-1*	11	RM332-RM116	RM116	16.70	33	0.50
	9311	*qPi93-3*	12	RM3246-RM7102	RM7102	7.41	16	0.49
162-8B	9311	*qPi93-2*	11	RM254-RM224	RM224	8.72	31	0.49
ARB52	9311	*qPi93-3*	12	RM3246-RM7102	RM7102	42.91	53	0.57
ARB94	9311	*qPi93-3*	12	RM3246-RM7102	RM7102	18.81	28	0.37
ARB50	9311	*qPi93-2*	11	RM254-RM224	RM224	16.28	25	0.30

in the RILs (P<0.01). All the skewed SSR markers were distorted to the parent 93-11, and most of the distorted loci were clustered on chromosomes 1, 3, 6, 10, and 11 (data not shown).

Identification of *R* genes

Using QTL Cartographer, we identified four major QTLs based on the disease resistance phenotype and genotype data of the tested RILs. Of them, three were from 93-11 and one was from Nipponbare. The resistant QTL from Nipponbare that conferred resistance to PO6-6 was named *qPiN-1*; it was mapped to chromosome 1 and was closely linked with SSR marker RM302. The locus accounted for 34% of the phenotypic variation. Two QTLs on chromosome 11 were identified, and these were derived from 93-11. The first QTL conferred resistance to CHE86, was named *qPi93-1*, and was mapped to the short arm of chromosome 11. *qPi93-1* was linked with SSR marker RM116 and explained 33% of the phenotypic variation. The second QTL was named *qPi93-2*, was mapped to the long arm of chromosome 11, and was closely linked to marker RM224. *qPi93-2* conferred resistance to 162-8B and ARB50 and contributed 31% and 25% of the phenotypic variation, respectively. The third QTL from 93-11 was identified on chromosome 12 and was named *qPi93-3*. *qPi93-3* was linked to the SSR marker RM7102, conferred resistance to three isolates (CHE86, ARB52, and ARB94) and accounted for 16, 53, 28% of the phenotypic variation, respectively.

Discussion

The elite hybrid rice restorer line 93-11 has been grown widely in China for many years and has been extensively used as a parent in breeding programs. For example, it is the restorer line for the famous two-line hybrid LYP9 (Dai et al. 1997; Yu et al. 2002). 93-11 is highly resistant to many Chinese *M. oryzae* isolates (Yang et al. 2009) and was reported to contain the *R* gene *Pi41* (Yang et al. 2009). In the current study, we identified three new *R* genes in 93-11 located on chromosome 11 and 12. *Pi93-1* is located on the short arm of chromosome 11 near blast *R* genes *Pi30* and *Pia* (Sallaud et al. 2003; Okuyama et al. 2011). *Pi93-2* is linked to the marker RM224 on the long arm of chromosome 11 where at least five blast *R* genes are located: *Pik* (Zhai et al. 2011), *Pik-m* (Ashikawa et a1. 2008), *Pik-p* (Wang et al. 2009), *Pik-h* (Sharma et al. 2009; Rai et al. 2011), and *Pik-s* (Fjellstrom, 2004). *Pi93-3* is linked to the marker RM7102 near the centromere region of chromosome 12 and near one of the most concentrated of blast *R* gene clusters. In this region, 14 *R* genes are mapped including *Pita* in cultivar K1 (Bryan et al. 2000), *Pita-2* in cultivar *PiNo.4* (Fjellstrom et al. 2004), and *Pi4* in Tetep (Yu et al. 1991). Fine-mapping of these genes will not only provide information on the relationship between these three new *R* genes and known genes but will also provide information on tightly linked markers; such information will enable marker-aided selection of these genes in rice breeding programs.

The R gene *qPiN-1*, which was detected in Nipponbare, confers resistance to *M. oryzae* isolate PO6-6 and is linked with SSR marker RM302 on chromosome 1, which is also where *Pish* and *Pi37* are located (Lin et al. 2007; Takahashi et al. 2010). *qPiN-1* may be *Pish* because Nipoponbare was reported to harbor *Pish* on chromosome 1 (Imbe and Matsumoto 1985) and because *Pish* confers resistance to PO6-6 (Takehisa et al. 2009). Fine-mapping of this gene in 93-11 should clarify the relationship between *qPiN-1* and *Pish*.

We found that the segregation ratio of resistant to susceptible plants to blast isolate ARB50 in the RILs does not fit into a 1:1 ratio (Table 2). A possible cause of the deviation is the segregation distortion in crosses between indica and japonica cultivars. It was reported that many loci in the progeny of indica-japonica rice hybrids showed segregation distortion (Harushima et al. 1996; Wang et al. 1994; Xu et al. 1997). We observed that the fertility of the F_1 plants of the Nipponbare/93-11 cross was quite low (about 10%). Low

fertility even existed in some of the F_2 and F_3 plants. Because of the relative large number of RILs (250) used in the inoculation with ARB50, we think that the segregation distortion may have no effect on the mapping of the resistance genes in the RILs to this isolate.

Materials and Methods

Plant Materials and Growth Conditions

A cross was made between 93-11 and Nipponbare for the development of the RIL population using the single-seed descent method. F_2 seeds were harvested from 15 F_1 plants for the development of the F_2 to F_7 RIL lines. A total of 259 F_7 RILs were obtained. After 20 seeds of each line were pre-germinated on 1/2 MS medium and kept at room temperature for 4 to 5 d, they were sown in a small plastic tray (20×20 cm) filled with sterilized soil (one RIL per tray). Plants were grown in a growth chamber at 23-26ºC, 80% RH, and 12-h light/12-h dark. When the plants were at three- to four-leaf stage (after 3 weeks in the growth chamber), they were used for DNA extraction and pathogen inoculation.

Isolate Selection, Fungal Growth, and Conidial Production

To identify isolates that showed different pathogenicity on the two parents, we screened 14 *M. oryzae* isolates from four countries (Table 1). Isolates were preserved on desiccated filter paper at -20°C. Dried filter papers containing fungal mycelia were placed on oatmeal agar plates for 7 d. The activated fungi were transferred to fresh medium and kept in an incubator at 25°C for 7 d under darkness and an additional 7 d under continuous fluorescent light. Spores were harvested in a water solution containing 0.02% Tween 20 and filtered through microcloth. The spore concentration was adjusted to 5×10^5 spores per ml with a hemacytometer before spores were applied by spraying (see next section).

Blast Inoculation and Disease Evaluation

The rice plants were spray inoculated according to a standard method with some modifications (Qu et al. 2006). For each RIL, one pot containing 15–20 plants (3 weeks old) was placed in a plastic bag and sprayed with 5 mL of the spore suspension using an airbrush with a pressure pump. Inoculated plants were kept in the bag that was placed in a big plastic container at 25°C, 80-90% humidity. After 24 h, the seedlings were removed from the bags and returned to the same growth chamber for an additional 5-6 d to allow disease development. Blast disease was evaluated 6-7 d after inoculation using a 0-5 scoring system (Zhou et al. 2006). The inoculation was repeated at least one time for each RIL, and the disease score was averaged from the two replications. If the results were different from the two inoculations, a third inoculation was carried out to confirm the disease phenotype.

Map Construction and Linkage Analysis of *R* gene

DNA was extracted using the method described by Tai and Tanksley (1990). A total of 131 simple sequence repeat (SSR) markers were used for linkage analysis, and these were evenly distributed at approximately 10-cM intervals on the 12 rice chromosomes. The primer sequences and positions of the SSR markers were obtained from the Gramene database (http://www.gramene.org). PCRs were performed as previously described by Liu et al. (2008) except that the reaction volume was changed to 25 μL. Amplified products were diluted 40-5000×, and 2 μL of the diluted product was added to 9 μL of formamide containing the LIZ-labeled size standard. PCR products from four primer pairs that had different size ranges and fluorophore labels were combined to determine the sizes of SSR alleles. The PCR products were run on an ABI 3730 DNA Analyzer according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Fragment sizing and SSR marker genotype analysis were performed using GeneMapper® software Version 3.7 (Applied Biosystems, Foster City, CA).

The SSR-based genetic linkage map was constructed with JoinMap 4.0 (Van Ooijen 2006) using the Kasambi mapping function. QTL Cartographer v 2.5 (Wang et al. 2012) was used to identify *R* genes conferring blast resistance using the composite interval mapping (CIM) method. The percentage of the total phenotypic variation explained by each locus and the additive effects were estimated with the same software. Tests were performed at every 2-cM interval on the linkage map, and cofactors were selected by forward/backward stepwise regression (Model 6) in QTL Cartographer. The significance threshold for CIM was determined by 300 permutation tests. In this study, the threshold value of LOD was 2.5. The phenotypic variation explained by a QTL conditioned by the CIM cofactors included in the model was calculated at the most likely QTL position.

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